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Real-time PCR Detection of Sorghum Ergot Pathogens *Claviceps africana*, *Claviceps sorghi* and *Claviceps sorghicola*

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Abstract

Sorghum ergot is a serious disease that has caused major losses in sorghum growing regions worldwide. *Claviceps africana,* originally reported from Zimbabwe, is now the most widely distributed species causing ergot in many countries including the United States of America, whereas both *C. africana* and *Claviceps sorghi* exist in India. A third species (*Claviceps sorghicola*) has been described causing sorghum ergot in Japan. As the three species show morphological similarities, a DNA-based assay is desirable for rapid identification in cases where ergot-infected sorghum is found by regulatory authorities. We designed PCR primers and probes from the intron 3 region of the β-tubulin gene (for *C. africana* and *C. sorghi*) and the intron 4 region of EF-1α (for *C. sorghicola*) and tested them by real-time PCR with purified DNA and ergot samples from the field and greenhouse. The primer and probe sets specifically amplified DNA from the respective species with a detection limit of c. 1 pg DNA. Genomic DNA from six other *Claviceps* species did not amplify in any of the three ergot species-specific assays. The assays we describe will provide useful tools for detecting sorghum ergot pathogens in seed and grain shipments and for determining which species are present in the samples, thereby aiding in the regulatory decision-making process.

Introduction

Ergot of sorghum is a serious and threatening disease which has caused substantial losses on a global scale (Frederickson et al. 1991; Bandyopadhyay et al. 1998). Sorghum ergot can be caused by three different species of *Claviceps*. While *Claviceps africana* is the species currently established in the United States of America, *Claviceps sorghi* exists in Africa and India along with *C. africana* (Bogo and Mantle 1999; Pažoutová and Bogo 2001; Johnson and Rajasab 2003; Muthusubramanian et al. 2006; Tooley et al. 2006; Johnson et al. 2008). A third species, *Claviceps sorghicola*, causes sorghum ergot only in Japan (Tsukiboshi et al. 1999).

Claviceps africana can move rapidly between continents. Sorghum ergot was only reported to exist in Africa and Asia prior to 1995 (Bandyopadhyay et al. 1998). It reached Australia in 1996 (Ryley et al. 1996) and was first observed in the western hemisphere in Brazil in 1995 (Reis et al. 1996). By early 1997, the disease had spread rapidly across South and Central America, the Caribbean, Mexico and the United States of America (Bandyopadhyay et al. 1998; Isakeit et al. 1998; Velasquez-Valle et al. 1998; Pažoutová et al. 2000).

Since the introduction of *C. africana* to the United States of America in 1997, sorghum ergot outbreaks have been sporadic (Workneh and Rush 2002, 2006; Prom et al. 2005; Workneh et al. 2006). Nonetheless, it has occasionally caused problems in particular regions. In 2005, ergot was found in a sorghum seed plot at the USDA-ARS Germplasm Introduction Research Unit in St Croix, Virgin Islands, and the seed was quarantined until identification of the pathogen as *C. africana* could be made. In the Texas panhandle, widespread damage occurred in hybrid seed production fields when *C. africana* first occurred in 1997 and since then it has continued to cause yearly seed production problems as well as occasional widespread epidemics (Workneh and Rush 2006; Workneh et al. 2006). Also in 2007, an outbreak of sorghum ergot occurred in sorghum breeders' plots in West Lafayette, Indiana (L. Dunkle, personal communication).

Claviceps africana can spread by various means including windborne spread of secondary conidia (Frederickson et al. 1993) and via seedborne inoculum and soil (Bhuiyan et al. 2002). The long-term viability of seedborne inoculum in particular makes sorghum ergot a disease of potential quarantine significance. The only ergot-causing species currently present in the United States and many other countries is *C. africana* (Odvody

et al. 2002). To prevent the introduction of species such as *C. sorghi*, which exists in Asia, into countries that currently harbour only *C. africana*, international seed exchange efforts must be monitored and rapid and accurate detection technologies employed.

Morphological differences exist among the three *Claviceps* species causing sorghum ergot (Frederickson et al. 1991; Tsukiboshi et al. 1999; Muthusubramanian et al. 2006). However, certain characteristics such as colony morphology and conidial dimensions overlap among the species, so detection methods based on characteristics other than morphology are desirable. DNA-based methods provide rapid, sensitive and specific detection for many fungal plant pathogens and currently polymerase chain reaction (PCR)-based methods are the most widely used methods (Schaad

Table 1
Isolates of *Claviceps* spp. used in this study

Species	Isolate	Origin	Host
Claviceps africana	Cls1	India	*Sorghum bicolor*
C. africana	Cls2	India	*S. bicolor*
C. africana	Cls3	India	*S. bicolor*
C. africana	Cla1	Australia	*S. bicolor*
C. africana	Cla2	Australia	*S. bicolor*
C. africana	Cla3	Australia	*S. bicolor*
C. africana	Cla4	US (College Station, TX)	*S. bicolor*
C. africana	Cla5	US (College Station, TX)	*S. bicolor*
C. africana	Cla9	US (Weslaco, TX)	*S. bicolor*
C. africana	Cla10	US (Weslaco, TX)	*S. bicolor*
C. africana	Cla7	US (Isabella, PR)	*S. bicolor*
C. africana	Cla8	US (Isabella, PR)	*S. bicolor*
C. africana	Cla12	US (Isabella, PR)	*S. bicolor*
C. africana	Cla34	US (PR)	*S. bicolor*
C. africana	Cla38	US (Virginia, NE)	*S. bicolor*
C. africana	Cla40	US (Virginia, NE)	*S. bicolor*
C. africana	Cla56	South Africa	*S. bicolor*
C. africana	Cla58	South Africa	*S. bicolor*
C. africana	Cla64	Mexico	*S. bicolor*
C. africana	Cla65	Mexico	*S. bicolor*
C. africana	Cla70	US (Georgia)	*S. bicolor*
C. africana	Cla77	US (Georgia)	*S. bicolor*
C. africana	Cla80	Japan	*S. bicolor*
C. africana	Cla83	Japan	*S. bicolor*
C. africana	Cla89	Zambia	*S. bicolor*
C. africana	Cla102	US (Kansas)	*S. bicolor*
C. africana	Cla103	US (Kansas)	*S. bicolor*
C. africana	Cla117	Zambia	*S. bicolor*
C. africana	Cla120	Zimbabwe	*S. bicolor*
C. africana	Cla122	Zimbabwe	*S. bicolor*
C. africana	Cla129	US (Florida)	*S. bicolor*
C. africana	Cla130	US (Florida)	*S. bicolor*
C. africana	ClaIN1	Andhra Pradesh, India	*S. bicolor*
C. africana	ClaIN17	Karnataka, India	*S. bicolor*
C. africana	ClaIN28	Maharashtra, India	*S. bicolor*
C. africana	ClaIN36	Rajasthan, India	*S. bicolor*
C. africana	ClaIN40	Utar Pradesh, India	*S. bicolor*
C. africana	ClaIN71	Gujarat, India	*S. bicolor*
Claviceps citrina	Ccit	Mexico	*Distichlis spicata*
Claviceps fusiformis	Clf1	Africa	*Pennisetum typhoideum*
C. fusiformis	Clf2	Africa (Zimbabwe)	*P. typhoideum*
C. fusiformis	Clf3	Africa (Zimbabwe)	*P. typhoideum*
Claviceps gigantea	Cgig	Mexico	*Zea mays L.*
Claviceps paspali	Cpas1	US (North Carolina)	*Paspalum sp.*
C. paspali	Cpas2	US (Georgia)	*Paspalum sp.*
Claviceps purpurea	Clp1	US (Montana)	*Hordeum vulgare*
C. purpurea	Clp2	E. Germany	*Secale cereale*
C. purpurea	Clp3	US (New Jersey)	*Spartina sp.*
Claviceps pusilla	Cpus1	Australia	*Botriochloa sp.*
C. pusilla	Cpus2	Australia	*Dicantium sp.*
Claviceps sorghi	IN6	Andhra Pradesh, India	*S. bicolor*
C. sorghi	IN10	Andhra Pradesh, India	*S. bicolor*
C. sorghi	IN11	Andhra Pradesh, India	*S. bicolor*
C. sorghi	IN32	Maharashtra, India	*S. bicolor*
C. sorghi	IN67	Maharashtra, India	*S. bicolor*
Claviceps sorghicola	Cjap1	Tochigi, Japan	*S. bicolor*
C. sorghicola	Cjap2	Tochigi, Japan	*S. bicolor*
C. sorghicola	Cjap3	Tochigi, Japan	*S. bicolor*
C. sorghicola	Cjap4	Tochigi, Japan	*S. bicolor*
C. sorghicola	Cjap5	Tochigi, Japan	*S. bicolor*
C. sorghicola	Cjap6	Tochigi, Japan	*S. bicolor*
C. sorghicola	Cjap7	Tochigi, Japan	*S. bicolor*

and Frederick 2002; Atkins and Clark 2004). A rapid and specific DNA-based assay is desirable to aid in discriminating *Claviceps* species when quick decisions must be made. A PCR-based method was developed for detection of *Neotyphodium* fungi, which are closely related to *Claviceps* species, in grass species (Dombrowski et al. 2006). A nested PCR assay was developed for detection of clavicipitaceous pathogens of rice and related grasses (Zhou et al. 2003). In addition, a PCR-based mating-type assay has been developed for members of the Clavicipitaceae (Yokoyama et al. 2004).

Earlier, we described primers for conventional PCR detection of *C. africana*, *C. sorghicola*, *Claviceps purpurea*, *Claviceps fusiformis* and *Claviceps paspali* based on the β-tubulin intron 3 region of chromosomal DNA (Tooley et al. 2001). This conventional PCR assay successfully identified *C. africana* as the pathogen causing the 2005 outbreak in the Virgin Islands. Real-time PCR (RT-PCR) technology offers several advantages over use of conventional PCR which include savings in labour and potential improvements in sensitivity, speed and versatility (Schaad and Frederick 2002; Schena et al. 2004). In this study, species-specific RT-PCR primers and probes were developed from the intron 3 region of the β-tubulin gene (*C. africana* and *C. sorghi*) or the EF-1α gene intron 4 (*C. sorghicola*). We describe the RT-PCR assays developed for specific and sensitive detection of the three *Claviceps* species which cause sorghum ergot.

Materials and Methods

Sources of cultures and DNA extraction

Claviceps isolates (Table 1) were obtained from a range of sources or isolated directly from diseased sorghum or grasses submitted to our laboratory (Tooley et al. 2000, 2001, 2006; Muthusubramanian et al. 2006) Cultures were maintained on potato dextrose agar (DIFCO; Voigt Global Distribution Inc., Lawrence, KS, USA) at 22°C in darkness. Total genomic DNA was extracted using a modified (CTAB) DNA extraction protocol (Taylor and Powell 1982) from 0.2 g lyophilized tissue grown in yeast-malt (YM) Broth (DIFCO). The DNA concentrations were determined using a spectrophotometer (Model ND-1000; Nanodrop Technologies, Wilmington, DC, USA). Species in addition to those causing sorghum ergot included *C. purpurea* (US and Germany), *C. fusiformis* (Africa), *Claviceps gigantea* (Mexico), *Claviceps citrina* (Mexico), *C. paspali* (US) and *Claviceps pusilla* (Australia).

Primers, probes and PCR conditions

Primers were designed from the β-tubulin gene intron 3 region or the EF-1α gene intron 4 (Tooley et al. 2001) using Primer Express v2.0 software (Applied Biosystems, Foster City, CA, USA). Primers were synthesized by Operon (Operon Biotechnologies, Inc., Huntsville, AL, USA). Dual-labelled fluorescent probes were labelled at the 5′ end with either the reporter dye 6-carboxylfluorescein (FAM) or CAL Fluor Orange 560 (CAL Orange) and labelled at the 3′ end with a Black Hole Quencher® (BHQ) (Biosearch Technologies, Novato, CA, USA) (Table 2). RT-PCR was performed using an ABI Prism 7700 Sequencer Detection System (Perkin Elmer/Applied Biosystems, Foster City, CA, USA) in a total volume of 25 μl containing 1 μl of DNA template. Cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 65°C for 1 min. The real-time PCR assay was optimized in multiple experiments by varying the concentrations of magnesium, primers, probes and annealing temperatures (data not shown). The final reaction components used were 500 nM of each primer, 1 μM of fluorescent probe and 1x TaqMan Universal Master Mix (Perkin Elmer/Applied Biosystems) with an additional 0.5 mM $MgCl_2$. The reaction mix was brought up to a final volume of 25 μl. Water was used in all reactions as a negative control.

Dilution series experiments

DNA was extracted twice from representative isolates of each *Claviceps* species being tested and quantified spectrophotometrically. Each quantified DNA sample was serially diluted, and two experiments containing two replications each were then performed with each dilution series, for a total of eight observations per species and DNA concentration.

Field and greenhouse samples

Field samples of *C. sorghi* were collected in India (Johnson and Rajasab 2003; Muthusubramanian et al.

Table 2
Primers and probes used for real-time PCR in discriminating three species of *Claviceps*

Target	Primer/probe	Sequence (5′–3′)	Length (bp)
Claviceps africana	CafrFb	TTGATCAAACATCCTCCTCTCCTCG	25
C. africana	BtCla2	TATGCTTGCACTCCCTTCGC	20
C. africana	BtafricanaCO	5′ CAL Fluor Orange 560 -(CCC TTA CAC ATC TAT TGT TAT GGG) BHQ-1 3′	24
Claviceps sorghi	BtSorgFa	GTTACCTACACATCTACCGTTGTGG	25
C. sorghi	BtSorgR2	CCAGAGGCCTATCGTATAGTTTAGCA	26
C. sorghi	CsorgFAM	5′FAM d(TGA GAC CTA GAG GAG TGC AAG CAT GTG T)BHQ-1 3′	28
Claviceps sorghicola	CjapF2	TCCTGCGAGCATGCCATAAGC	21
C. sorghicola	CjapR3	CTGTATTGATTCGTTCATGTTGTTCAC	27
C. sorghicola	CjapCO2	5′d CAL Fluor Orange 560 –(CCT GGC CCC TCT TTT TGA CCG C) BHQ-1 3′	22

Table 3
Cycle threshold (Ct) values for isolates of *Claviceps africana*, *Claviceps sorghi*, *Claviceps sorghicola* and other *Claviceps* species used in real-time PCR analysis

	Ct value[a]		
	C. africana primers and probe	*C. sorghi* primers and probe	*C. sorghicola* primers and probe
C. africana			
Cls1	27.54 ± 0.16	>40 ± 0[b]	>40 ± 0[b]
Cls2	25.77 ± 0.18	>40 ± 0	>40 ± 0
Cls3	29.47 ± 0.31	>40 ± 0	>40 ± 0
Cla1	28.76 ± 0.14	>40 ± 0	>40 ± 0
Cla2	28.71 ± 0.27	>40 ± 0	>40 ± 0
Cla3	27.88 ± 0.36	>40 ± 0	>40 ± 0
Cla4	26.35 ± 0.28	>40 ± 0	>40 ± 0
Cla5	28.39 ± 0.29	>40 ± 0	>40 ± 0
Cla9	27.65 ± 0.30	>40 ± 0	>40 ± 0
Cla10	26.41 ± 0.13	>40 ± 0	>40 ± 0
Cla7	28.02 ± 0.62	>40 ± 0	>40 ± 0
Cla8	27.54 ± 0.84	>40 ± 0	>40 ± 0
Cla12	26.32 ± 0.06	>40 ± 0	>40 ± 0
Cla34	26.01 ± 0.08	>40 ± 0	>40 ± 0
Cla38	26.84 ± 0.20	>40 ± 0	>40 ± 0
Cla40	28.17 ± 0.43	>40 ± 0	>40 ± 0
Cla56	28.26 ± 0.22	>40 ± 0	>40 ± 0
Cla58	27.50 ± 0.25	>40 ± 0	>40 ± 0
Cla64	28.94 ± 0.17	>40 ± 0	>40 ± 0
Cla65	27.63 ± 0.35	>40 ± 0	>40 ± 0
Cla70	26.58 ± 0.65	>40 ± 0	>40 ± 0
Cla77	27.31 ± 0.52	>40 ± 0	>40 ± 0
Cla80	27.70 ± 0.68	>40 ± 0	>40 ± 0
Cla83	26.68 ± 0.44	>40 ± 0	>40 ± 0
Cla89	28.01 ± 0.47	>40 ± 0	>40 ± 0
Cla102	26.91 ± 0.98	>40 ± 0	>40 ± 0
Cla103	28.70 ± 0.97	>40 ± 0	>40 ± 0
Cla117	24.85 ± 0.28	>40 ± 0	>40 ± 0
Cla120	26.21 ± 0.34	>40 ± 0	>40 ± 0
Cla122	27.01 ± 0.59	>40 ± 0	>40 ± 0
Cla129	27.80 ± 0.44	>40 ± 0	>40 ± 0
Cla130	28.95 ± 0.22	>40 ± 0	>40 ± 0
ClaIN1	30.90 ± 0.22	>40 ± 0	>40 ± 0
ClaIN17	25.86 ± 0.18	>40 ± 0	>40 ± 0
ClaIN28	27.47 ± 0.11	>40 ± 0	>40 ± 0
ClaIN36	25.09 ± 0.09	>40 ± 0	>40 ± 0
ClaIN40	25.24 ± 0.07	>40 ± 0	>40 ± 0
ClaIN71	26.97 ± 0.15	>40 ± 0	>40 ± 0
C. sorghi			
IN6	>40 ± 0	29.46 ± 0.20	>40 ± 0
IN10	>40 ± 0	32.45 ± 0.22	>40 ± 0
IN11	>40 ± 0	28.48 ± 0.20	>40 ± 0
IN32	>40 ± 0	28.41 ± 0.25	>40 ± 0
IN67	>40 ± 0	28.24 ± 0.16	>40 ± 0
C. sorghicola			
Cjap1	>40 ± 0	>40 ± 0	22.77 ± 0.58
Cjap2	>40 ± 0	>40 ± 0	24.78 ± 0.58
Cjap3	>40 ± 0	>40 ± 0	27.57 ± 0.35
Cjap4	>40 ± 0	>40 ± 0	29.34 ± 0.28
Cjap5	>40 ± 0	>40 ± 0	29.72 ± 0.65
Cjap6	>40 ± 0	>40 ± 0	28.55 ± 0.56
Cjap7	>40 ± 0	>40 ± 0	28.35 ± 0.32
Other Claviceps species[c]	>40 ± 0	>40 ± 0	>40 ± 0
Negative control	>40 ± 0	>40 ± 0	>40 ± 0

[a]Data are mean values of two experiments each containing two replications per treatment ±standard error ($n = 4$).
[b]No fluorescence was detected at 40 cycles of PCR amplification when tested at a concentration of 1 ng DNA.
[c]Other species are listed in Table 1.

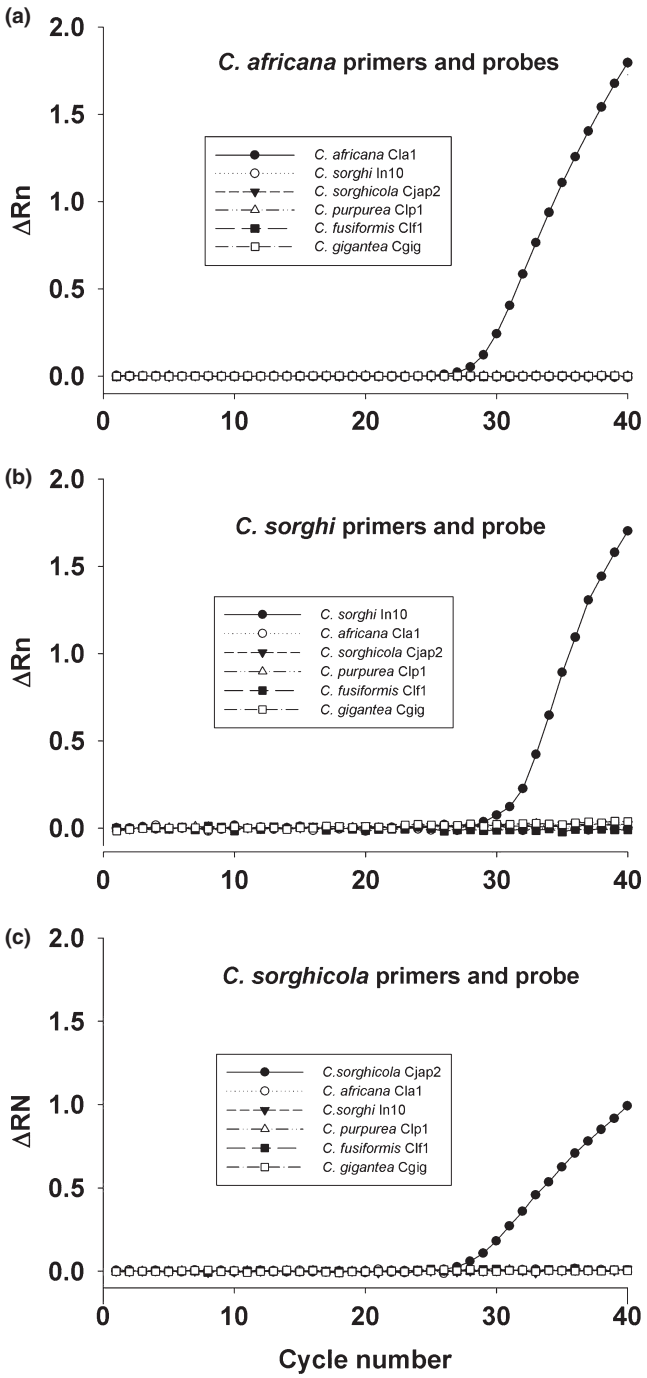

Fig. 1 Representative real-time PCR amplification profiles using (a) *Claviceps africana*, (b) *Claviceps sorghi*, and (c) *Claviceps sorghicola* primers and probes

2006). The India 2005 samples (sclerotia and infected florets – see Table 5) were collected 14 December 2005 from volunteer sorghum (variety unknown) growing in a pigeon pea field ca. 20 km southeast of Gulbarga, India. The field sample 'India 2000' (infected florets) was the source of *C. sorghi* isolate NAP7 (Pažoutová and Bogo 2001; Muthusubramanian et al. 2006). Samples were maintained at −20°C until used in the assay.

Samples of *C. africana*-infected material from Indiana were collected from male-sterile lines at the Agronomy Center for Research and Education in West Lafayette, Indiana in October 2007. Texas samples

were collected by Dr Gary Odvody at Corpus Christi, Texas in June 2007.

Male-sterile sorghum line ATx623 was grown from seed in a level BSL-3 containment greenhouse until flowering heads suitable for inoculation developed. A conidial suspension was prepared by placing infected field samples in distilled water for several minutes with mild agitation to allow release of conidia into the water. Heads of sorghum line ATx623 at the flowering stage were then dipped in the suspension and covered with a quart-sized plastic bag. After 48 h in the greenhouse, the bags were removed and symptoms were observed after a further 48–72 h. To produce sorghum heads infected with isolates (such as those of *C. sorghicola*) which were received as pure cultures, a conidial suspension was made from a culture growing on potato dextrose agar by using a cotton swab to dislodge conidia into distilled water. Flowering heads of ATx623 were then dipped into this suspension and bagged as indicated previously. DNA for use in the rtPCR assay was extracted from field samples and greenhouse-inoculated material using a MO BIO PowerSoil™ DNA Kit (MO BIO Laboratories, Inc. Carlsbad, CA, USA) according to the manufacturer's instructions.

Data analysis

Data acquisition and analysis were performed using the TaqMan data worksheet and software according to the manufacturer's instructions (Applied Biosystems). The cycle threshold (Ct) values for each reaction were calculated automatically by ABI Prism sequence detection software (ver. 1.6.3) by calculating the PCR cycle at which the reporter fluorescence exceeded two times the background.

Results

Only the target species amplified with the primers and probe developed for that species. Positive RT-PCRs to detect *C. africana*, *C. sorghi* and *C. sorghicola* showed Ct values ranging from 22.77 to 32.45, while negative reactions failed to cross the threshold by the end of 40 cycles (Table 3). For *C. africana*, 38 isolates chosen to represent diverse geographical regions were tested and were strongly positive (Table 3). Five isolates of *C. sorghi* and seven isolates of *C. sorghicola* tested strongly positive with their respective primer and probe sets. Fig. 1 shows representative amplification profiles for each set of primers and probes. No amplification was obtained in any of the three species-specific assays when DNA from six additional *Claviceps* species was used as template (Table 3, Fig. 1). These included *C. purpurea*, *C. fusiformis*, *C. gigantea*, *C. ci-*

Table 4
Cycle threshold values and standard errors obtained in dilution series experiments using spectrophotometrically quantified DNA of *Claviceps africana*, *Claviceps sorghi*, and *Claviceps sorghicola*

DNA concentration	*C. africana*	*C. sorghi*	*C. sorghicola*
1 ng	26.74 ± 0.14[a]	28.36 ± 0.26	24.22 ± 0.11
100 pg	28.64 ± 0.80	31.35 ± 0.23	28.01 ± 0.30
10 pg	33.80 ± 0.28	35.72 ± 0.64	31.76 ± 0.16
1 pg	38.75 ± 0.83	38.52 ± 0.59	35.22 ± 0.29
100 fg	>40 ± 0	>40 ± 0	38.55 ± 0.46

[a]Standard error ($n = 8$).

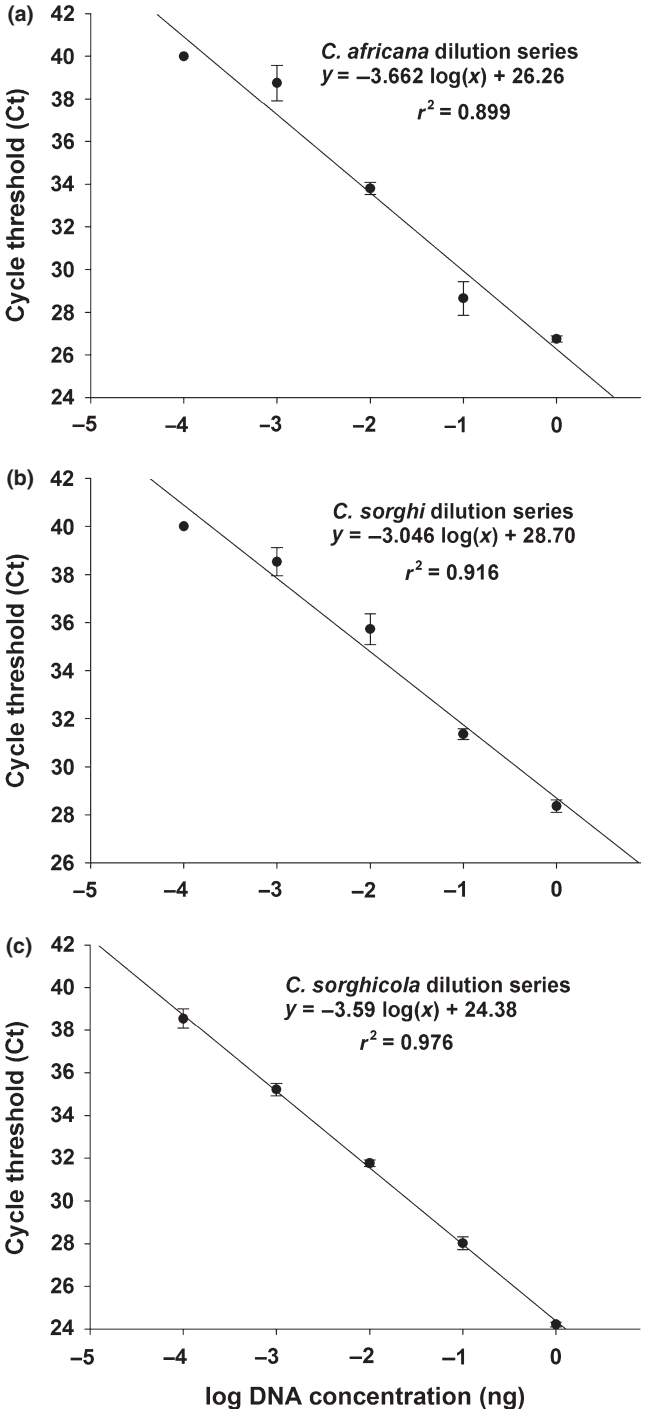

Fig. 2 Standard curves of cycle threshold values calculated from serial dilutions of DNA from (a) *Claviceps africana* (isolate Cla1), (b) *Claviceps sorghi* (isolate IN11), and (c) *Claviceps sorghicola* (isolate Cjap1) with standard error bars indicated

trina, *C. pusilla* and *C. paspali* from varied geographical sources.

Results of replicated DNA dilution series experiments revealed the detection limits of our assay. Table 4 shows the mean Ct value for each species and DNA dilution along with its standard error based on eight replications performed. Fig. 2 shows the graphical relationship between log (DNA concentration) and Ct values. The threshold limit of detection was 1 pg genomic template DNA for *C. africana* and *C. sorghi*, but somewhat lower for *C. sorghicola* as some Ct values <40 were obtained at the 100 fg level (Table 4).

Positive Ct values obtained from analysis of field and greenhouse samples collected in different years and locations ranged from 23.48 to 30.07 (Table 5), whereas negative samples showed Ct values above 40 cycles. In each case, samples could be clearly identified as having been infected by one or more of the three target *Claviceps* species. The India 2006 sample was tested positive for both *C. africana* and *C. sorghi*, indicating that it was the result of a mixed infection.

Discussion

Methods used in the past to detect ergot fungi or the alkaloids they produce have included light microscopy (Peace and Harwig 1982), seed washes (Sharma 1990), high performance liquid chromatography (Rottinghaus et al. 1993), mass spectrometry (Rowan and Shaw 1987), and competitive ELISA (Shelby and Kelly 1992). PCR methods offer many advantages over other methods including greater sensitivity, shorter assay times and high specificity to the target species (Schaad and Frederick 2002).

The real-time (RT-PCR) assay described here can discriminate among the three species of *Claviceps* that cause ergot of sorghum. The assays were based on the intron 3 region of the β-tubulin gene (*C. africana*, *C. sorghi*) or the EF-1α gene intron 4 (*C. sorghicola*). The assays were successful in detecting *Claviceps* species from infected greenhouse and field material, including sclerotia of *C. sorghi* from the field in India, and each assay can be completed rapidly. Ergot-causing *Claviceps* species can be rapidly identified with the described assays so that decisions about quarantine of seed or other materials can be made in a timely fashion.

In the assay with field material, the MO BIO PowerSoilTM DNA Kit for extracting DNA from field samples that were suitable for rtPCR provided more consistent results than methods described earlier (Tooley et al. 2001) which involved microcentrifugation and resuspension of samples in 1X PCR buffer.

The level of sensitivity we obtained was 1 pg of genomic DNA, which is less sensitive compared with some other described real-time PCR assays for fungal plant pathogens. Yan et al. (2008) found that the lower limit of detection was 0.4 pg of purified DNA in a real-time PCR assay for *Cladosporium fulvum* based on the β-tubulin gene. One hundred-fold greater sensitivity was obtained using primers based on the sequence of a microsatellite region. An assay for *Phialophora gregata* based on the intergenic spacer region of nuclear rDNA (Malvick and Impullitti 2007) showed a detection limit of 50 fg of genomic DNA. A PCR assay described for *Neotyphodium* endophytes by Dombrowski et al. (2006) based on the tubulin 2 gene had a detection limit of 5–50 pg DNA, which is less sensitive than the detection limit obtained in our assay. However, Zhou et al. (2003) found a detection limit of 2.3 pg target DNA when using conventional, non-nested PCR to detect clavicipitaceous pathogens *Ustilaginoidea virens* and *Ephelis japonica* but much greater sensitivity (2.3–23 fg target DNA) for nested PCR. Thus, even though less sensitive than some other reported real-time PCR assays, our assay proved sufficient for detecting multiple species of *Claviceps* in field material.

We have shown the assays we describe to be useful when performed individually to determine which of the three sorghum ergot-causing *Claviceps* species may be present in a particular sample. The assay will also be a useful tool in characterizing pathogen populations prevailing among various host populations in areas where several pathogen species coexist. A future goal

Table 5
Cycle threshold (Ct) values for ergot-infected sorghum field material, greenhouse-inoculated sorghum or uninfected sorghum

Sample[a]	*Claviceps africana* primers and probe	*Claviceps sorghi* primers and probe	*Claviceps sorghicola* primers and probe
Field samples			
Indiana 2007 infected florets	25.02 ± 0.20[b]	>40 ± 0[c]	>40 ± 0
Texas 2007 infected florets	25.16 ± 0.17	>40 ± 0	>40 ± 0
Kansas 1999 infected florets	27.05 ± 0.11	>40 ± 0	>40 ± 0
Florida 1999 infected florets	25.61 ± 0.44	>40 ± 0	>40 ± 0
India 2005 sclerotium	>40 ± 0	23.48 ± 0.18	>40 ± 0
India 2005 infected florets	26.08 ± 0.07	29.40 ± 0.14	>40 ± 0
India 2000 (NAP7) infected florets	>40 ± 0	30.07 ± 0.24	>40 ± 0
Greenhouse-inoculated samples			
Japan, *C. sorghicola* (Cjap3)	>40 ± 0	>40 ± 0	29.08 ± 0.07
Japan, *C. sorghicola* (Cjap4)	>40 ± 0	>40 ± 0	24.38 ± 0.12
Uninfected sorghum head	>40 ± 0	>40 ± 0	>40 ± 0

[a]Samples were collected from naturally infected sorghum fields at the indicated location, or obtained via greenhouse inoculation using isolates supplied by colleagues as described in Materials and Methods.
[b]Ct value. Data are means of four observations (two experiments with two replications each).
[c]No fluorescence was detected at 40 cycles of PCR amplification.

is the development of a multiplex assay in which primers and probes for different target species would be combined in a single RT-PCR, potentially making the assay more rapid and efficient when screening for multiple *Claviceps* species within the same sample.

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